STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

THE HISTORY OF STAINING

For about five years a series of articles has been running in this journal on the History of Staining. This series is now practically complete. The July issue contained an article on the history of vital staining which brought that subject up to date except that one special field was omitted. This special field, the preservation of supravitally stained material, is now treated in a separate paper which is contained in the present issue.

This paper now published completes the series except for a few biographical sketches that are to be included of some of the early workers who made most important contributions to the subject. As soon as these sketches are drawn up, it is planned to reprint the whole series of papers on the History of Staining in book form and to put the book on sale.

ENLARGING THE SCOPE OF THIS JOURNAL

Readers of Stain Technology will have noticed that recent numbers have included several papers on microtechnic that have only indirect bearing on staining. There are two papers in the present number of this same character. It has seemed wise to include such articles, as there is no journal in the United States exclusively devoted to microtechnic. The matter of including these papers was approved at the last Executive Committee meeting of the Stain Commission, and in recognition of this policy it was then decided that beginning with the January, 1933, number this journal is to be called "Stain Technology and Microtechnic."

It is intended not only to include papers on new methods but comprehensive resumés of procedures in given fields. A beginning in this line has already been made by the paper by R. H. Wetmore in the April, 1932, number of the journal. This paper on The Use of Celloidin in Botanical Technic has now called forth a similar contribution printed elsewhere in this issue by G. L. Walls on the same method in zoölogical technic. Readers' attention is called to the fact that if

they desire such papers as these in separate form, for teaching purposes or laboratory use, reprints can be ordered either direct from Stain Technology or from the Wistar Institute at the University of Pennsylvania, Philadelphia.

—H. J. Conn

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the July number of this journal.

Stains Certified June 1 to August 31, 1932*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene violet	NMv 2	....	As constituent of tetrachrome blood stain	June 11, 1932
Erythrosin B	LEr 2	71%	As histological counterstain	July 9, 1932
Methyl green	NG 10	....	In histology and in Pappenheim stain	July 18, 1932
Azure C	NAc 2	....	In histology, and as constituent of blood stains	July 20, 1932

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

HISTORY OF STAINING

Methods for the Preservation of Supravitally Stained Material

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The methods used in the preparation of sections from tissues of animals which have been vitally stained require very few modifications of the ordinary histologic methods. In the case of supravital staining, on the other hand, the preservation of permanent sections is more difficult. Supravital dyes diffuse into living cells very rapidly, hence their staining capacity; but they likewise leave the cells rapidly, and therefore are usually lost during the fixation and washing of tissues. Several methods have been proposed for the preservation of supravitally stained tissues, all depending upon the relative solubilities of the dyes used.

Apparently the first method used for the preservation of supravitally stained cells was that of Lewis (1922). In his experiments explants from chick embryos of from five to ten days incubation were grown in Locke-Lewis solution. They were subjected to a weak solution of Janus green, neutral red, or both, until stained. The cultures were then fixed with iodine vapor by placing a small flake of pure iodine on the bottom of the hollow ground slide. In these experiments both the Janus green and the neutral red were discolored, but both were preserved in their morphologic aspects.

McJunkin (1925) reported the preservation of supravitally stained tissues by the same method which he had used for peroxidase material. The principal features of this procedure were to inject into the tissue immediately after death a saline solution saturated with neutral red; to introduce a piece of tissue not over 3 mm. in thickness into a Zenker's solution with formalin but without acetic acid; to dehydrate with acetone and pass thru benzol into paraffin. The sections were counterstained in hematoxylin or methylene blue.

In connection with his studies on the origin of the mononuclear cells, McJunkin (1925, 1926 a and b) has reported slight modifications of the method given above, in respect to the fixation in Zenker's fluid and the dehydration.

A similar method has been used by Cash (1926). He injected the dye intravenously instead of directly into the tissues. His

method was developed primarily for the study of the tubercle organism in tissues and consequently the decolorization of the carbol fuchsin stain had to be carefully controlled in order to prevent decolorization of the neutral red. The characteristic features of his procedure are: Injection of the neutral red saline solution into an ear vein; after cutting the sections, removing the paraffin and passing thru acetone into water, they are first treated with iodine, and then stained with carbol fuchsin, decolorized and counterstained with methylene blue as customary in staining acid-fast organisms.

Sherwood (1926) has also described a method for the preservation of supravitally stained material. Her method differs from those of McJunkin and Cash in that it is carried out on the slide. The cells (blood, exudates or tissue cultures) are stained supravitally on the slide, the coverslip is fixed for ½ to 1 hour at 38° to 40° C. in the Zenker-formol mixture of Fischer. After washing and drying, the preparations are stained with Wright's stain or methyl green.

Chlopin (1927) used a mixture of Zenker-formol and Champy's fixative for studying vital staining in insects, amphibia and invertebrates. In some instances he injected the neutral red; in others he kept the animals in solutions of the dye until properly stained. After supravital staining, the tissues (or entire animal when possible) were fixed in Champy's fluid or 8% ammonium molybdate for from 1 to 48 hours, then placed in osmic acid, washed for 24 hours in running water, dehydrated quickly in absolute alcohol, cleared in xylol and embedded in paraffin. He considered thionin and methylene blue the best nuclear stains to use following neutral red supravitally.

Gardner (1927) has modified McJunkin's procedure in several particulars. The neutral red in saline solution is injected into the trachea and the whole animal incubated 5 to 30 minutes at 37°C. Special detailed directions are given for preparing the Zenker-formol solution which the author recommends. After fixation, the blocks of tissue are washed under vacuum in fresh Zenker's solution without formalin or acetic acid. They are then washed in water and passed thru graded alcohols into benzene and then into paraffin. After cutting the sections and removing the paraffin, each must be handled individually. The slides are passed thru alcoholic iodine, and then after 95% alcohol, are counterstained in Harris' hematoxylin. They are then dehydrated, cleared, and mounted in balsam.

Scott (1928) preserved neutral red and Janus green in films of blood and bone marrow by drying them in a vacuum desiccator for 3 hours and then washing thoroly with anhydrous ether, subsequently clearing in xylol and mounting in balsam. Scott notes that this

method is particularly good for bone marrow, as it permits the removal of all the fat.

Chuin (1930) has used the neutral red method to study changes in the digestion vacuoles in schyphistomes. They are stained with neutral red or with a mixture of neutral red and methylene blue in sea water, observed under the microscope and later fixed by running a small amount of fixative under the edge of the coverslip, while one aspirates on the opposite side with blotting paper. After the object has been fixed, it can be dehydrated while being studied under the microscope. After dehydration, one carefully raises the coverslip and applies a drop of Canada balsam. The fixative employed to maintain neutral red is the Bouin-Holland solution in which the acetic acid has been replaced by trichloricetic acid. In dehydrating, amyl alcohol and 95% ethyl alcohol are used as the first change. They are replaced by pure amyl alcohol, which is left for a considerable period of time, as its action is very slow.

Taft and Ludlum (1930) have developed a method for staining nervous tissue with argyrol which should be considered in this connection. It does not produce any distortion of tissues. Fresh brain tissue is placed in 10% argyrol for 6 to 24 hours, then washed in distilled water. Small fragments are placed on a slide in a drop of water and a coverslip floated over them. They can be dehydrated and cleared in the usual way.

Forkner (1930) has reported a modification of Cash's method which is more elaborate, but which he states gives more constant results. For this purpose, he has devised special apparatus for injecting the dye and describes the procedure in greater detail than can be repeated here. Injection is thru the left ventricle in small animals and the carotid in larger ones. The small blocks of stained tissue are fixed, then dehydrated in alcohol, followed by acetone, then passed thru xylol into paraffin. After cutting the sections, removing the paraffin, and passing into alcohol, counterstaining is performed with alcoholic iodine.

MacCarty (1930) has developed a method for the study of malignant tissues which depends upon the reactions of frozen tissues of fresh material to polychrome methylene blue. His method is used primarily for the differentiation of malignant and non-malignant lymph nodes. Lymph nodes are sectioned at 5 to 20μ on a freezing microtome, care being taken to avoid large crystallization of cellular fluids. The sections are placed in salt solution, then stained in Unna's polychrome methylene blue from 1 to 3 seconds, washed in salt solution and mounted in isotonic Brun's glucose.

Hu (1931) points out that, in the methods previously described for the preservation of supravitally stained material, there are certain difficulties. These are the necessity for rapid handling to prevent the loss of neutral red and the inevitable loss of some of the dye, even when all the directions are followed carefully.

To avoid these difficulties, he fixes the blocks of tissue in a special fluid, a Zenker solution to which neutral red dissolved in formalin is added. Dehydration is carried out in absolute alcohol containing a certain amount of mercuric chloride which is saturated with neutral red. The bits of tissue are then run thru xylol into paraffin and sectioned. After removing the paraffin the section may be mounted immediately, or may be counterstained as follows: Wash off the xylol with the same solution as used for dehydrating, then pass into an aqueous solution of mercuric chloride saturated with neutral red, immerse in methylene blue dissolved in the same aqueous solution; and finally, wash off the methylene blue with the aqueous mercuric chloride neutral red solution, dehydrate, clear, and mount.

Hu claims that with this method there is neither distortion of the tissues nor loss of the neutral red. Advantage is taken of the fact that neutral red is only slightly soluble in aqueous or alcoholic solutions containing mercuric chloride but that sufficient dye can be brought into solution to check further removal of the neutral red from the tissue.

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EOSIN B

W. C. Holmes,[1] C. G. Melin[2] and A. R. Peterson[3]

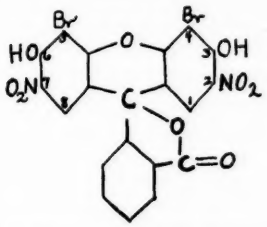
Contribution No. 208, from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Abstract.—**The two known isomeric dibromodinitrofluoresceins have been prepared in a fairly pure state, and their absorption spectra determined.**

Commercial samples of eosin B are not 4, 5-dibromo—2, 7-dinitrofluorescein, as stated in dye indices. They are mixtures which contain other bromonitro derivatives of fluorescein as well as dibromodinitro derivatives.

The color acid method provides a substantially reliable means of determining actual dye content with commercial samples of the dye, but the reduction method may prove decidedly misleading.

Both Rowe (1924) and Schultz (1930) list eosin B as the alkali salt of 4, 5-dibromo—2, 7-dinitro fluorescein.



4, 5-dibromo—2, 7-dinitro fluorescein

It was first prepared and described by Baeyer (1876) and was soon afterward marketed under the name of safrosin. Recent dye indices list some seventeen manufacturers and an equal number of trade designations. It still finds some employment in the industries and also finds application in biological staining.

Spectrophotometric examinations of the dye at this laboratory have indicated decided variations in the characteristics of different

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samples, and the "color acid" (1923) and reduction (Knecht and Hibbert, 1925) methods of determining dye content have occasionally yielded widely divergent results. This investigation was undertaken to obtain criteria for the identification of the dye, to secure further information on the character of market supplies, and to determine the relative reliability of different methods for evaluating dye content.

A considerable variety of methods have been described for the synthesis of dibromodinitrofluorescein, including the nitration of (1) dibromofluorescein and (2) tetrabromofluorescein; the bromination of (3) dinitrofluorescein, and (4) tetranitrofluorescein, and (5) the practically simultaneous nitration and bromination of fluorescein itself. While it is doubtless possible to obtain a dibromodinitrofluorescein by each of these methods, it is evident that the same dye will not be obtained in each instance.

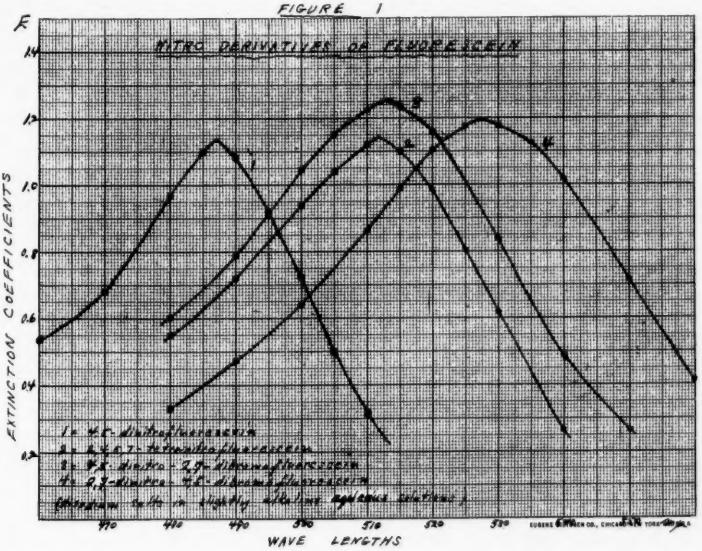
It has been thoroly established that the 4 and 5 positions in the fluorescein molecule are invariably the first in which substitution occurs (Hewitt and Woodforde, 1902). Altho the latest edition of Schultz (1930) states that 4, 5-dibromo—2, 7-dinitrofluorescein may be prepared by brominating dinitrofluorescein, Hewitt and Woodforde have shown that the product so obtained is 2, 7-dibromo—4, 5-dinitrofluorescein, the salts of which are decidedly yellower and less desirable in hue than are those of its isomer. It is also doubtful if the nitration of tetrabromofluorescein (eosin) would yield the particular isomer desired, and it appears certain that Mühlhäuser's method for the preparation of the dye from fluorescein (cited by both Rowe and Schultz) cannot yield a pure 4, 5-dibromo—2, 7-dinitrofluorescein since some measure of nitration must occur at the 4 and 5 positions under its provisions before any possibility for bromination therein is provided.

EXPERIMENTAL

4, 5-DIBROMO—2, 7-DINITROFLUORESCEIN

Both Baeyer (1876) and Hewitt and Woodforde (1902) succeeded in brominating tetranitrofluorescein and obtaining a 4, 5-dibromo—2, 7-dinitrofluorescein which appeared identical with that which they obtained by nitrating dibromofluorescein. We were able to prepare tetranitrofluorescein of excellent purity very readily by Baeyer's method. It gave an absorption maximum at 512mμ in alkaline aqueous solutions (Fig. 1) and one at 523mμ in an alkaline mixture of equal volumes of water and alcohol. Preliminary trials of the Hewitt and Woodforde method of brominating this compound indicated that more effective conditions would be required for the introduction

of two bromine atoms. Since dibromofluorescein appeared a more promising intermediate for the preparation of the dye, no further effort was made to utilize tetranitrofluorescein for the purpose.



The degree of bromination and nitration in these products, and in those referred to subsequently, was determined in the following manner: Bromine values were obtained by fusions with sodium peroxide in Parr bombs, employing the Pringsheim (1904) technic. Results obtained in this way were found to be in good agreement with others obtained by the Carius method. Nitration was estimated by means of quantitative reduction with titanous chloride (Knecht and Hibbert, 1925). Each compound requires two hydrogen equivalents for reduction and, in addition, six hydrogen equivalents for each substituent nitro group.

A sample of 4, 5-dibromofluorescein, which was obtained from a commercial source, had a melting point of 264° to 265° C. and was represented as being "pure." Analysis indicated approximately 10% overbromination. The bromination of fluorescein at this laboratory by Baeyer's method (1876) yielded a product which proved to be 9% overbrominated.

The attempt to nitrate the latter material in accordance with the directions supplied by Hewitt and Woodforde (1902) resulted in a product which consisted principally of dibromomononitrofluorescein. A process described by Mühlhäuser (1887), however, in which dibromofluorescein is heated for several hours with powdered sodium nitrate in glacial acetic acid, was found to be effective. The an-

alytical data on the product indicate approximately 6% undernitration and 3.5% overbromination. (The formula is $C_{20}H_8Br_2N_2O_9$. Bromine content: theoretical 27.56%; found 28.50%, 28.56%. Normal $TiCl_3$ consumption: theoretical 24.14 cc. per gm.; found 22.62 cc., 22.75 cc.)

The dye gave an absorption maximum of 528mμ in alkaline aqueous solution (Fig. 1) and of 533mμ in alkaline 50% alcohol.

It was found possible to obtain products which had the same absorption spectra by mixing a dilute alcoholic solution of dibromofluorescein with half its volume of concentrated nitric acid and allowing the mixture to stand over night, and also by dissolving dibromofluorescein in fuming nitric acid and drowning the charge after a few minutes.

It may be noted that overnitration may occur under favoring conditions. Dibromofluorescein and even tetrabromofluorescein are converted completely into tetranitrofluorescein by the protracted action of fuming nitric acid.

2, 7-DIBROMO—4, 5-DINITROFLUORESCEIN

4, 5-Dinitrofluorescein was prepared from fluorescein by the procedure suggested by Hewitt and Woodforde (1902). Analysis indicated 5% nitration over the dinitro stage. The dye had an absorption maximum of about 487mμ in alkaline aqueous solution (Fig. 1) and one of about 494mμ in alkaline 50% alcohol. These values appear somewhat remarkable. The corresponding constants for fluorescein itself are 490mμ and 496mμ. It would hardly be anticipated that the introduction of nitro groups in the 4 and 5 positions of the fluorescein molecule would exert a slightly bathychromic effect on the hue of the dye.

The dinitrofluorescein was brominated in accordance with Hewitt and Woodforde's directions. The analysis of the dye obtained indicated slightly less than 10% overbromination and a corresponding degree of undernitration. (The formula is $C_{20}H_8Br_2N_2O_9$. Bromine content: theoretical, 27.56%; found, 30.21%, 30.12%. Normal $TiCl_3$ consumption; theoretical, 24.14 cc.; found 21.83 cc., 21.71 cc.)

The dye gave an absorption maximum of 513mμ in alkaline aqueous solution (Fig. 1) and one of 518mμ in alkaline 50% alcohol.

COMMERCIAL SAMPLES OF EOSIN B

The only data available in the literature which could throw light on the character of commercial samples of eosin B are the spectroscopic measurements recorded in Formanek and Grandmougin's tables (1913). Six samples were examined of which two proved

somewhat different from the remaining four. In aqueous solutions their absorption maxima ranged between 519.2mμ and 521.5mμ.

Only two eosin B samples were available at this laboratory in amounts sufficient for investigation. They were precipitated in the form of their color acids and washed thoroly to obtain material for analysis. Bromine values were obtained as described, and nitrogen values determined by the Kjeldahl method as modified for total nitrogen.[4]

An eosin B of textile grade was found to contain 33.38% bromine (found 33.18%, 33.60%, 33.30%; theoretical 27.56%) and 2.74% nitrogen (found 2.77%, 2.71%; theoretical, 4.83%). These values indicate the probability of a mixture of tribromomononitrofluorescein and dibromomononitrofluorescein rather than a dibromodinitro derivative. The absorption maximum was 521mμ in alkaline aqueous solution and 526mμ in alkaline 50% alcohol.

An eosin B marketed for biological staining was found to contain 26.56% bromine (found, 26.32%, 26.80%; theoretical 27.56%) and 4.45% nitrogen (found, 4.48%, 4.43%, 4.43%; theoretical 4.83%). These values indicate the probability of dibromodinitrofluorescein with 3.5% underbromination and 8% undernitration. The absorption maximum was found to be 518mμ in alkaline aqueous solution and 524mμ in 50% alcohol. Previous small samples of stain from the same manufacturers have had similar but slightly different spectrophotometric characteristics.

It is obvious that none of the samples of eosin B examined by either Formanek or ourselves can be 4, 5-dibromo—2, 7-dinitrofluorescein, since their absorption spectra differ from that of the latter very greatly. They also differ in a corresponding degree from 2, 7-dibromo—4, 5-dinitrofluorescein. Their absorption alone is insufficient for their characterization. It is necessary to determine bromine and nitrogen values to obtain a more definite clue to their character. They may consist principally of mixtures of the two isomeric dibromodinitrofluoresceins, as appears to be the case in the instance of the stain sample examined by ourselves, or of mixtures of various other bromonitro derivatives, as seems evident in the case of the textile dye which we analyzed.

ANALYSIS OF EOSIN B

Eosin B is marketed as its disodium salt, and commercial samples contain moisture and inorganic salts or other impurities. The prin-

[4]Official and Tentative Methods of the Association of Official Agricultural Chemists. Washington, 1925.

cipal, and ordinarily the only, demand made upon the dye analyst is that of determining "actual dye content." The dye content of dye samples in general is usually determined by means of reduction with titanous chloride (Knecht and Hibbert, 1925). Eosin B is readily reduced by titanous chloride, and excellent checks are obtained in duplicate reductions, but it will be evident, in view of the general character of commercial samples of the dye, that the method must necessarily prove a very unreliable means of evaluating its dye content.

This may be illustrated by a hypothetical case. We may assume that a given dye sample contains 80% of actual dye, but that the dye consists of equal proportions of mononitrodibromofluorescein and dinitrodibromofluorescein instead of consisting entirely of the latter. In such an instance it may be calculated readily that the indicated dye content by the reduction method will be 64.8% instead of 80%.

On the other hand, the reliability of the "color acid method" (Holmes, 1923) for determining the total dye content of such samples is but little impaired by reason of the variation of their character from that assumed by the analyst. Dye content is calculated by applying a "conversion factor" to the determined percentage of color acid, and the conversion factor for any given dye differs but slightly from those of the related dyes which may probably also be present. In the hypothetical instance cited above, for example, the calculated dye content would be 79.9% instead of 80%. The error involved in the erroneous basis of calculation would amount to only 0.1%.

Altho the absolute accuracy of the color acid method has not been completely demonstrated in the instance of eosin B, considerable evidence of its substantial reliability has been obtained. The method is known to give remarkably accurate results with erythrosin (Holmes, 1923) and eosin.

The determination of total dye content by the color acid method may be supplemented by spectrophotometric examination and by bromine and nitrogen determinations upon the precipitated color acid if a more searching examination of the sample is desired. The bromine and nitrogen values obtained will supply the necessary data for revising the conversion factor employed in calculating total dye content and enable the analyst to report reliable dye contents even in instances in which the sample may be radically abnormal.

It will probably be seldom, however, that these additional data will afford the analyst a complete clue to the precise character of the sample. Should the spectrum conform closely to that of one of the isomeric dibromodinitrofluoresceins, and the bromine and nitro-

gen values be found in close agreement with theory, the identity of the dye could safely be considered as established. With products of a heterogeneous character, such as have reached this laboratory hitherto, such data will serve only to indicate the probable general character of the sample.

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A NEW TIME-SAVING DEVICE FOR EMBEDDING IN PARAFFIN

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Abstract.—**A simple device to be used in place of the usual paper boxes or watch crystals used for embedding in paraffin in the preparation of histological sections is described. Short lengths of cooled rubber tubing are placed on a glass surface, which has previously been painted with glycerin, and are filled with melted paraffin. The rubber tubing sections are best cut by stretching the tubing on a wooden rod and cutting while the wood is turning in a lathe.**

It is obvious that any time-saving device in routine laboratory procedures is always welcome. With this in mind, we wish to report a simple substitute for the folded paper boxes or watch crystals which technic books prescribe for embedding specimens in paraffin. Guyer[1] devotes a paragraph and Lee,[2] a whole page to the description of the preparation and use of the paper boxes or trays generally used to enclose the specimens. McClung[3] advocates the watch glass method, which certainly has advantages over the paper box one, but we have found that even this procedure is difficult for some students to follow. Particularly if temperature conditions are not uniform is it difficult to secure a well built-up block of paraffin.

Our device is very simple and inexpensive. We use short sections of rubber tubing set on a glass slide, a piece of plain window glass an inch or more square, or the tube may be set into a watch crystal. The glass is rubbed with glycerin and the tubing cooled before using. The tubing is set upon the glass and warm paraffin run into the rubber container. The paraffin in contact with the glass and rubber cools slowly and hardens. Meanwhile, the specimen is placed and oriented in the center. Blowing a little air over the top will form a congealed film. The entire preparation is lowered carefully but quickly into cold water. As the paraffin cools it shrinks but the rubber tubing yields on all sides. Thus the shrinkage is not all from the top and so does not cause the "center dip" commonly resulting in solid con-

[1]Guyer, M. F. Animal Micrology. Univ. of Chicago Press. 1926. (See p. 37).

[2]Lee, Bolles. Microtomist's Vade-Mecum. Blakiston's, Philadelphia. 1928. (See p. 74–75).

[3]McClung, C. E. Handbook of Microscopical Technique, Paul Hoeber, New York. 1929. (See p. 15).

tainers. When fully hardened, the block is removed from the slide and the rubber separated from the round, well-formed block of paraffin, which may then be labelled. The tubing and glass are again ready for use.

This method has been used in an undergraduate laboratory class and has proven most successful. It is also a great saving of time in routine work.

The size of the rubber tubing and the length of the sections to be used will vary with the size of the piece to be embedded. The thickness of the rubber may vary; the only essential being that the tube shall maintain its shape. We are providing for our students at the present time and would recommend tubing of 10 and 20 mm. in diameter, and sections cut 7 and 14 mm. in length. We find that a good way to cut the rubber tubing so that the sections will have smooth, straight edges is to place it on a round rod of wood, which is then put into a lathe. The tubing is now cut readily into sections with a sharp knife while the wood is turning.

We claim the following advantages for the device:

1. It forms smooth and regular blocks.
2. It prevents the melting of one edge and the spreading of the paraffin, a difficulty often experienced in the watch-glass method.
3. It allows easy orientation.
4. It permits uniform and rapid cooling.
5. It causes no "central dip."
6. It is inexpensive.
7. *It saves a great deal of time.*

MALLORY'S CONNECTIVE TISSUE STAIN FOLLOWING HEMATOXYLIN

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ABSTRACT.—**The following combination of hematoxylin with Mallory's connective tissue stain is useful in bringing out nuclei as well as in differentiating tissue:**

Slightly overstain in Mayer's hematoxylin (50 g. potassium alum and 0.2 g. sodium iodate added to 1 liter 0.1% aqueous hematoxylin). Wash; and stain 30 seconds to 1 minute in 0.04% aqueous acid fuchsin. Stain 4 minutes in: 0.5 g. anilin blue and 2 g. orange G dissolved in 100 cc. of 1% aqueous phosphomolybdic acid. Pass thru 95% alcohol to absolute; clear in xylol and mount in balsam.

Mallory's connective tissue stain is used extensively to differentiate tissues but delineates nuclei poorly. Combined with a good nuclear stain, as hematoxylin, it should prove a very valuable routine procedure. The author has tried such a combination and developed, what he believes, a successful method.

Mayer's and Harris' hematoxylins were tried, the former giving the better results. The Mallory "A" solution was slightly modified, a greater dilution being employed. This stain follows well after Zenker, Bouin or formalin fixation; with the last named, however, the sections must be mordanted in Bouin's fixing fluid for 15 to 20 minutes before staining. The results with this method were quite satisfactory—nuclei stained deep purple; cytoplasm pink; secretions, cartilage, collagenous and reticular fibres light blue; erythrocytes orange or red; and muscle pink. It was quite easy to see the nuclei of smooth muscle cells by this method, ordinarily impossible with the Mallory stain.

After developing this method, a search thru the literature revealed that at least two other workers had attempted a similar technic, Ukai (1926) and Brilmeyer (1928 and 1929). Ukai used hematoxylin to precede a complicated modification of Mallory's stain which required three solutions and several hours to stain. Brilmeyer's preparations, which required at least two hours to stain, showed the nuclei colored a "rich red"; apparently the hematoxylin was in a slightly acid condition.

The following procedure, which leaves the nuclei in the normal

deep purple color, affords a better contrast to the pink cytoplasm and a marked saving in staining time.

1. Slightly overstain in Mayer's hematoxylin prepared in the following manner:
   To 1 g. of hematoxylin in a liter of water add 50 g. of potassium alum and 0.2 g. sodium iodate. Dissolve and filter.
2. Wash in tap water.
3. Pass into diluted Mallory "A":
   Acid fuchsin, 0.04% aqueous solution; 30 seconds to 1 minute.
4. Pass into Mallory "B":

Anilin blue	0.5 g.
Orange G	2.0 g.
Phosphomolybdic acid, 1% aqueous sol.	100 cc.

   4 minutes.
5. Wash and differentiate in 95% alcohol.
6. Dehydrate in absolute alcohol.
7. Clear in xylol and mount in balsam.

This technic was originally worked out using National Aniline acid fuchsin and MacAndrews and Forbes' hematoxylin (obtained thru the National Aniline Company), while the anilin blue and orange G were Grübler's products. Later, upon the suggestion of the chairman of the Biological Stain Commission, certain other samples of domestic dyes were compared with the foreign dyes. Two samples of acid fuchsin (certification Nos. CR-1 and NR-10) which had been obtained from the Coleman and Bell Company and from the National Aniline Company respectively, two samples of anilin blue (CK-2 and NK-2) and two samples of orange G (CO-3 and NO-3) obtained from the same two companies were tried in the above technic. Far better results were obtained with the domestic anilin blue and orange G than with the Grübler dyes. The colors proved to be more brilliant and the differentiation more clear-cut than when the imported stains were used; in fact, the slides stained with the latter were drab in comparison with those in which the domestic stains were used. The best staining combination in this instance proved to be the Coleman and Bell acid fuchsin with the National Aniline orange G and anilin blue.

Attempts to check Brilmeyer's hypothesis, that either the acid fuchsin or the phosphomolybdic acid converted the hematoxylin to a "rich red" color, resulted in proof to the contrary. Slides, stained in Mayer's hematoxylin, were placed into various solutions for relatively long periods of time; *e. g.*, an acid fuchsin solution for 15 minutes, a 1% phosphomolybdic acid solution for 3½ hours, or a Mallory "B"

solution for 3 hours. In no instance did the hematoxylin assume the "rich red" coloration. The use of a 0.04% acid fuchsin instead of the usual 0.2% solution permitted a finer differentiation between cytoplasm and nucleus.

I am indebted to Dr. H. M. Kingery for his helpful aid, particularly for suggesting the dilution of the Mallory "A" solution and the use of Bouin's as a mordant.

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THE HOT CELLOIDIN TECHNIC FOR ANIMAL TISSUES[1]

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ABSTRACT.—**The writer's modifications of the Jeffrey celloidin technic, as adapted to animal histology, are given in detail. No claim is made that the methods are new; but because animal tissues will withstand treatment which would ruin those of plants, the writer has been able to introduce many short-cuts into the original hot celloidin method. It is felt that these modifications deserve serious consideration even from those zoölogists to whom time and trouble are important factors in the preparation of histologic material. It is also pointed out that the celloidin method has several advantages over the paraffin technic that are seldom realized.**

The recent appearance of the valuable paper by Wetmore[3] impels the writer to record the details of the hot celloidin method of embedding as now employed after six years of experience in sectioning animal material. Dr. Wetmore has simplified to some extent the original Jeffrey method; but even with a reduction in the number of solutions employed, the method may still appear too complicated, to a zoölogist reading Wetmore's paper, to ensure it a fair trial in his hands. Animal tissues, even the most delicate, will withstand treatment which would ruin plant tissues; consequently, the writer has found it possible to introduce many short-cuts into the original hot celloidin method, and believes that in its present form as used by him it deserves serious consideration even from those zoölogists to whom time and trouble are important factors in the preparation of class or even of research material. Altho the "embedding" portion of the hot technic is not so rapid as with paraffin, the later processes are faster, easier, and more fool-proof; the embedding itself represents a vast saving of time over the ordinary cold method, and permits as thin sections as does paraffin. Serial sections are admittedly troublesome to cut, but the total time consumed in preparing serial slides is actually less than in the case of paraffin. Moreover, most zoölogists will admit that they very often cut serial sections where such are quite unnecessary.

[1]Reprints of this paper can be obtained at 25c each either from the Wistar Institute at Philadelphia or the Biological Stain Commission at Geneva, N. Y.

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[3]Wetmore, R. H. The use of celloidin in botanical technic. Stain Techn., **7**, 37. 1932.

Some seldom-realized advantages of the hot celloidin method over the paraffin technic are listed as an appendix to the description.

Materials Needed

Warm-box:

Any incubator or embedding oven may be used, provided its heating element is not an open flame or exposed incandescent metal. It is set at about 60°C.

Embedding bottles:

Embedding is carried on in bottles, their size depending on the amount of tissue to be placed in each bottle. For most purposes a two-ounce pomade bottle is best. This size will comfortably handle up to four pieces of tissue ⅜ inch on a side. For very large pieces a three or four-ounce bottle is necessary, not so much for its greater capacity as for its larger aperture.

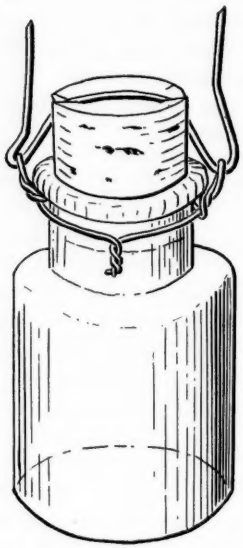

Fig. 1. Embedding bottle, showing method of wiring stopper.

Choose a make of bottle that has a thick lip; this is important. The bottles used must have bottoms of uniform thickness; discard any that have thick and thin places, or bubbles in their sides. Twist two ears in a piece of No. 18 soft iron wire at such points that when the wire is bent around the neck of the bottle the ears will be diametrically opposite. Twist the ends of the wire tightly together and cut off ¼ inch from the bottle.

For holding in the cork against the pressure of the solvent vapor use 5 inch lengths of No. 16 copper wire (copper is less brittle than brass and need not be renewed during an embed; the diameter being greater than that of an equally flexible brass wire, it cuts into the cork to a much less extent). When wiring up a bottle (see Fig. 1) bend about 1 inch of the wire nearly double with small pliers and hook it thru one ear of the bottle. Bend the wire over the cork and pass it thru the other ear; grasp this end with pliers and pull down, out and up with a single motion. The copper wire should run *parallel* to any cracks there may be in the top of the cork. The same wire and cork will serve for a whole embed if the cork can just be started

into the bottle at first. Do not attempt to use either wire or cork for another embed.

Solutions:

Solutions should be kept in wide-mouthed glass-stoppered bottles. After pouring from or into a stock bottle, wring the stopper in well and it will come out very easily later. Avoid cork stoppers as they tend to freeze, and will then break before they will release. Ordinarily, 500 cc. bottles are large enough for a single person running thru research material or blocks for a small class. Very small bottles should not be used even if little embedding is being done, as the per cent change in the strength of solutions due to evaporation from opened bottles is greater. It is advisable to have extra-large or duplicate bottles for the thinnest and thickest solutions, as these are consumed while the others are not.

The concentrations used are 2, 4, 6, 10, and 14%. The 4% and 14% solutions may be omitted under certain circumstances—the 4% is not necessary if the tissue is easily infiltrated, and the 14% is needed only with large or dense blocks.

Photographers' "soluble cotton" is perfectly suitable for the stock solutions, and costs only one-tenth as much as other nitro-celluloses; there are some solid impurities in the cheaper material, but these sink to the bottom of the stock bottle and do no harm. Regardless of the material used for the stocks, Mallinckrodt's "pyroxylin" should be purchased for use in the thickening process, to be described later.

The solvent is a 50–50 mixture of ether and either 96% or absolute alcohol. The nitro-cellulose will dissolve most readily if soaked in the alcohol 24 hours before the ether is added. The 14% solution will be perfectly homogeneous after 72 hours if stirred occasionally to break up lumps (invert the bottle frequently, also) and embedding may be commenced as soon as the 2% solution is ready.

Other reagents and instruments:

Chloroform, not necessarily of high quality, is used for hardening. After hardening, blocks are stored in a 50–50 mixture of 96% alcohol and glycerin.

Instruments needed for the complete technic are: Sliding microtome, preferably of inclined-plane type; a *sharp* knife with both sides plane and a relatively thin back (Spencer) and at least 4 inches long; flat-surfaced wood or fibre block for microtome; sable brush with ¾ inch tuft; small section lifter; dissecting needle in handle; a few slender insect pins; safety razor blade; Syracuse glasses or small Stender dishes; and a couple dozen spring type clothes-pins. An

important implement is a small spoon which will easily enter the embedding bottles; such a spoon can be quickly made from a piece of heavy copper wire with a peen hammer. A medicine dropper is helpful for producing currents in the rinsing dish when staining sections.

Embedding

Dehydrate the tissue to at least 96% alcohol, preferably to absolute; material destined for celloidin may be left over night in either of these fluids, as no excess hardening effect is manifest from their extended use, as with paraffin. Transfer to an embedding bottle (the writer ordinarily fixes and dehydrates in the latter) and add 2% celloidin to ¼ inch or so above the pieces; wire up and place upright in the warm-box.

After 24 hours infiltration will be complete unless the piece of tissue is more than 1 inch in diameter. The high pressure developed within the bottle, together with the higher viscosity of the heated celloidin, make for very rapid penetration as compared with cold solutions. Do not fear to leave material for so long a time at such a temperature. In the paraffin method, it is the *hot paraffin*, not the *heat per se*, which prohibits such infiltration-times because of the resultant brittleness of the block. Tissue can be left indefinitely in celloidin of any strength without harm (a great convenience on holidays, week-ends, and vacations); the writer has cut perfect 5μ sections of tissues which had purposely been left in the warm-box for eight months.

Allow the bottle to cool on a wooden table. Do not hasten the cooling by placing on soapstone or holding under running water, as strains will be set up in the glass which will surely result in the bottle breaking in a future embed. If cooled upon wood, bottles will never break from this cause.

Open the bottle and pour off the 2% solution. Do not restore it directly to the stock, as it is diluted, but, if enough bottles are running to make it worth while, pour the 2% solution into a dish and allow it to evaporate. When dry, cut into strips and use for making up stocks.

Pour on the next solution, wire up, and replace in the warm-box for a half-day; change to successively higher solutions at half-day intervals (morning and late afternoon or evening).

Thickening

When the 14% solution is put in, it should just cover the tissue pieces (unless the latter are *very* small) and at the same time some

pyroxylin should be added. The correct amount is for experience to teach, but ordinarily one strip, cut in two transversely, is enough for a 2-ounce bottle. The pyroxylin pieces should be completely immersed and so arranged that they prevent the tissue from moving down the slope of the bottom and getting into the angle between bottom and side.

Do not expect the pyroxylin to go into solution; its purpose is to rob the 14% solution of so much solvent that it becomes very thick. Allow the mass to thicken in the warm-box overnight or 24 hours. Here is the one point in the entire technic that requires judgment; when the bottle is cool, the solution should *barely flow*. If it flows freely, it is because insufficient pyroxylin was added; replace in warm-box for another half-day or, if time is more precious than pyroxylin, add a bit more of the latter in future. The success of sectioning depends upon the perfection of the thickening. If the mass is less viscous than the above standard, the resultant block will rarely cut thinner than 5μ. If the mass is too thick, it is difficult to keep the tissue properly surrounded with celloidin when removing it for hardening. (If the beginner finds he has added so much pyroxylin that the solution becomes solid—it will then be milky—a little of the 14% solution added, and a return to the warm-box for half an hour will set things right).

Hardening

With the spoon, scoop out each piece of tissue separately with a globular mass of celloidin solution around it. Scrape from the spoon, with the needle, into about an inch of chloroform in a 4-ounce wide-mouthed bottle. The instant the mass touches the liquid chloroform a tough skin forms upon it; if necessary, a clean needle is thrust thru this skin to press the tissue into the middle of the globule. Gas bubbles need not be squeezed out unless greater than $\frac{1}{8}$ inch in diameter, as they cause no inconvenience in sectioning.

Leave the blocks in chloroform until they sink. Remove and quickly trim to a cube with the razor blade, leaving no more than $\frac{1}{16}$ inch of celloidin around the tissue at its thickest part. Transfer to a second bottle of clean chloroform. When the block sinks in this, it is done and is transferred to storing fluid in a vial. After a few minutes, it may be cut, or can be stored for years. Blocks stored for more than a few weeks, before or after cutting sections from them, should be put in vials with clean corks and the latter sealed against creepage of the glycerin by dipping the end of the vial in 6% celloidin.

In the case of very large or very delicate tissues which could not easily be scooped out after thickening, fill the embedding bottle with chloroform and allow to stand until the mass is firm on sides and bottom; allow access of the chloroform to the sides of the mass by passing a needle or a long Bard-Parker knife down the side of the bottle to the bottom and working it around the inside of the bottle, back to the starting point. When the mass is firm, break the bottle into a dish of chloroform, trim the block, and store after it has sunk.

Blocks may be left indefinitely in chloroform, but more than 24 hours has no further hardening effect, and small blocks are thoroly hardened in much less time. The two chloroforms employed for hardening are used repeatedly.

The thick mass left in the embedding bottle is scooped out, or better, pulled out in one piece after standing over night, and is dried down for use in making up stocks or for thickening. It is best cut to convenient size for thickening if not thoroly dry when cut. The residue which clings to the glass can be freed cleanly and pulled out in one piece if the bottle is filled with water and allowed to stand for a few minutes.

Sectioning

Blot the block with a cloth and cut a thin slice with a *clean* razor blade from the face which is to go against the fibre block in the microtome. With the handle of the needle put a drop of 10% celloidin on the fibre and quickly smear it around. Place the tissue quickly and firmly on this smear and paint the latter liberally with a brush dipped in chloroform. As soon as the chloroform evaporates, flood the knife and the block with 96% alcohol and proceed to cut. (The knife should slant at a long angle to its line of travel. Its under surface should be in a nearly horizontal plane, the edge a trifle lower than the back; experiment with these angles.)

Cut away the block in thick slices until the point is reached where sections are wanted. Flood the knife again and dip the brush in a dish of alcohol. Hold it horizontally close to the block so that alcohol is held between brush and block by capillarity. The brush should not rest firmly on the block or it will be cut. Pull the knife across, and if the brush is lifted slightly without lateral movement it will guide the section smoothly up onto the knife. Improper handling of the brush may make the sections roll, but no harm is done as they will unroll themselves when transferred to a dish of alcohol. Do not think, however, that the brush can be dispensed with; its function is not to keep the sections flat but to permit their being cut very thin. Their thinness depends upon the consistency of the tissue and matrix,

the sharpness of the knife, and the skill of the operator with the brush.

The *hot* celloidin matrix is glass-clear; parts of the block can be seen clearly before they are reached in sectioning, so that if only a portion of the section is really wanted; *e. g.*, a single organ in a section of an entire small animal or large embryo, the block can be trimmed pyramidally as soon as that organ is reached so that smaller sections, containing only that organ, are being cut; they can obviously be gotten much thinner. This economizes greatly in slides and covers and in time spent in staining, etc. Symmetrical material can be oriented precisely during the cutting process; simply mount a trial section in counterstain and examine under the microscope, then readjust the microtome accordingly. The block, if found to be worthless due to poor choice of material or fixative, can be discarded at this point—another saving over paraffin.

Series may be prepared by any of the methods described for cold celloidin sections or by Dr. Jeffrey's well known method, which permits transfer to stain almost immediately after the sectioning process is finished.

Staining and Mounting

Non-serial sections are handled in the same dish in which they were put when cut. If all are to be stained alike the various fluids are poured or pipetted on and off (except where complex stains are used such as Mallory's and Flemming's triples, Ehrlich-Biondi, etc., when the sections must be transferred individually or a few at a time from dish to dish). It is unnecessary to pass thru any intermediate solutions between 96% alcohol and water, in either direction, and a mere dip and rinse is enough in any alcohol or in the clearing fluid. When transferred from alcohol to either water or the clearing fluid, the sections will flatten out and shoot about on the surface; when they come to rest they should be submerged with the flat of the needle, else their upper surfaces may dry. Counterstaining with very rapid stains, such as phloxine, is best done on the section lifter, the stain being dropped on and drained off when its work is done. When an opaque stain is to be used and the staining time is measured in fractions of a minute, as with Mallory's triple[4] the section should not

[4]The writer has found that with hot celloidin sections the staining times for Mallory's triple stain are greatly reduced. Solutions A, B, and C of the original form are used for 30 seconds, 2 minutes, and 45 seconds respectively. From solution C the sections are dipped in water and then rinsed gently by pipetting in a dish of 96% alcohol until no more stain is seen to leave the clear periphery of the section; they are then dipped in absolute alcohol mixed with chloroform, and are cleared.

be released in the dish as it may not be recoverable at the proper time. Before placing in such stains, draw the section up onto a slip of stiff paper with the needle; rest the paper on the table and press an insect pin vertically thru the clear rim of the section until it just penetrates the paper. The end of the paper strip bearing the section and pin is now immersed in the stain, and the section can be transferred back to water at any instant.

Celloidin absorbs some stains, but rarely shows them under the microscope; this effect is much less marked with "hot" sections than with "cold" ones.

When dehydrating the stained sections, absolute alcohol can be used if it contains 5% of chloroform to destroy its solvent action. Benzol is the supreme clearer for this method; benzol-balsam is also excellent because of the more rapid drying. Sections need stay in benzol only long enough to permit the slide and cover to be wiped clean; they need be only optically clean as no adhesive is used. In mounting, transfer the section to the slide with a drop of benzol and orient it with the needle. Holding the balsam tube ready, stand the slide on end to drain off the excess benzol, and when the section is on the point of drying, add balsam and cover. Press the cover down with the handle of the needle until there is no appreciable amount of balsam above or beneath the section, then clamp the preparation with a clothes-pin to complete the flattening. The slide may be examined at once, even with oil-immersion, but the pin should be left on overnight and the excess balsam around the rim of the mount removed two or three days later with chloroform.

Miscellaneous Points

A very convenient means of identifying tissues thruout the process is afforded by the small, numbered metal tacks sold for marking window-screens and their proper locations, etc. Numbers 1 to 50, in aluminum, are obtainable from mail-order houses at less than one cent per tack; higher numbers are available in brass. The tissue is assigned a number and the proper tack is simply changed from cork to cork thru the processes of fixation, dehydration, embedding, hardening, and final storage. This procedure is more convenient than the use of paper labels and tags, which may come off or be rendered undecipherable by wetting.

The embedding process can be lengthened as much as is thought necessary for exceptionally large or impervious pieces, each solution being used for 24 hours or longer.

If forced to leave work temporarily while sectioning, wrap a piece of mulch paper soaked in storing fluid around the block; it will keep thus for some time.

The sections may stay in alcohol indefinitely before staining, and those not stained may be stored with the block.

Round cover glasses are decidedly preferable to square ones. If, when a section is ready to mount, it should be found that its edges have curled as sometimes happens when the block is of poor consistency or the sections have been in aqueous stains too long, place it on the slide with the curl up; after adding the balsam press the cover down quickly with a little lateral motion toward the curl or a circular motion if the curl is extensive around the edge of the section, and the rush of balsam toward the edge of the cover will flatten the curl perfectly. Clean-cut folds in the clear celloidin outside of the tissue do no harm unless the section is very thick.

Avoid Euparal as a mounting medium as it dissolves celloidin.

In trying a new stain, never use it for as long as is recommended for paraffin sections; halve the time for the first trial and increase gradually if necessary. Hot celloidin sections stain much more quickly than paraffin sections of the same thickness.

Keep stains in low Stender dishes; use Syracuse glasses for alcohols, water and clearer, and renew their contents frequently.

Sections can often be transferred from dish to dish draped over the needle, thus minimizing the time of the operation and the mixing of reagents; the lifter must be used, however, for thick or extremely thin sections.

Do not attempt to cut sections thinner than 10μ until your skill is such that you get every section at that thickness. The proper use of the brush is not learned in a minute or in an hour.

Some Advantages of Hot Celloidin Over Paraffin

1. Celloidin will handle anything organic; everything from *Spirogyra* to anthracite coal has been cut at 5μ in celloidin.

2. *Celloidin produces no artefacts.* There is absolutely no shrinkage or swelling; hence, if delicate connections, intercellular bridges, etc., exist in the fixed tissue they are faithfully preserved in the finished slide.

3. Celloidin does not pass cell membranes; hence, minute cytological structures are not subjected to the invasion of a viscous substance.

4. In cutting, there can be no telescoping of the section as with paraffin (as the latter is usually cut), with the consequent disruption

of delicate parts. One does not *chisel* bread—one *slices* it. Paraffin can be cut without telescoping only by the laborious water-on-the-knife method of Huber, in which a sliding microtome is employed with the knife at a long angle and flooded with water; Huber's method forbids ribbons and is more tedious than the cutting of celloidin.

5. The sectioning of celloidin is independent of temperature, humidity, and atmospheric electricity.

6. Tissues are not rendered hard and brittle by xylol, for none is used.

7. Limy material can be decalcified *after* embedding, thus avoiding the disruptive effect of gas bubbles in unsupported tissue.

8. Once the tissue is embedded, the process is much faster. Hundreds of sections can be stained and dehydrated at once in a small dish, and mounted as fast as slides can be wiped to receive them. There is no fuss with tricky albumen, no dangerous heating, no waiting for slides to dry before staining, no risk in using the slide as soon as the cover is on it.

9. Old, faded sections, sections on broken slides, etc., can be dissolved off, restained and remounted any number of times. A valuable section can be destained at any time and restained in some other way to bring out other details.

10. Because of the absolute transparency of the matrix, material can be oriented precisely for sectioning; the orientation can be checked exactly during the actual cutting process by mounting trial sections in alcoholic counterstain.

11. Embryos, hollow objects, etc., which have been sectioned to the median line can then be mounted *in toto* for topographical studies under the binocular.

12. Since the celloidin still supports the cells in the finished slide, there can be no crushing or displacement by the cover-glass. A 5μ celloidin section remains 5μ thick. The importance of this for vertical measurements in quantitative work, and for detailed reconstructions, is obvious.

13. Because of the above and of the optical properties of the celloidin matrix, the cells appear bathed in light and have more depth. More can be seen in a celloidin section than in a paraffin section of the same thickness.

14. Celloidin permits the use of Mallory's triple stain, whose value as a diagrammatic differentiator for class slides can hardly be exaggerated. The Mallory stain is most unsatisfactory on paraffin sections, but gives a splendid result with celloidin, for which it was designed. The staining time with celloidin is much reduced; a

finished Mallory slide can be obtained ten minutes after mounting the block in the microtome.

15. Examination of trial sections during cutting makes possible the discard of poor material without waste of time, and the high visibility of the contents of the block makes it unnecessary to cut any sections which are not actually wanted for finishing. Paraffin must be cut "blind."

16. Time tolerations are great—an important point with a class in microscopic technic or a commuting investigator. There is no last minute rushing to embed before the laboratory is locked up. One is not torn between the necessities of staying in hot paraffin long enough to get infiltration and of getting the material cooled before it is ruined.

Bear in mind that any technic is more formidable in print than in fact. If this treatment seems lengthy, it is because the writer has tried to make it so complete that anyone can adopt the method and get perfect results without needing a user of the method at his elbow. The only objection the writer has ever heard offered to the method is that it is slower than paraffin; but surely no research worker should value his time more than his confidence that his preparations tell him the truth.

The writer's own research material—the eyes of vertebrates—is as heterogeneous and refractory as any animal material, and his satisfaction with the hot celloidin method is such that he expects never to use paraffin again; he would alter that familiar dictum to read: "Celloidin if you care; paraffin if you don't."

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

ALEXANDER, ELEANOR G. Improved staining techniques for the demonstration of non-acid-fast tubercle bacilli and granules. Science, **75**, 197–198. 1932.

Two counterstaining methods for the sharper differentiation of acid-fast and non-acid-fast bacilli are described. A 10-days old strain of avian tubercle bacillus grown on glycerin agar with either 10% of normal horse serum or 10% immune rabbit serum was used. The smears were stained for 5 minutes by the Ziehl-Neelson method, decolorized with 3% acid alcohol, and rinsed under tap.

Method 1. Counterstain with Löffler's methylene blue. Add immediately 6 to 8 drops of 0.05% NaOH, agitate gently and let stand for 2 to 3 minutes. Wash under tap, dry and examine. The contrast between blue and red is striking.

Method 2. Flood the smear with 8 drops of a 1% aqueous crystal violet. Add immediately 6 to 8 drops of 5% $NaHCO_3$, agitate gently and let stand 2 to 3 minutes. Wash under tap, apply Gram's iodine for 2 minutes, wash, and decolorize 20 to 30 seconds with a mixture of equal parts of acetone and 95% alcohol; wash and dry. The non-acid-fast bacilli appear violet. In addition, the granules in the red acid-fast and the violet non-acid-fast bacilli stand out as violet-black bodies. (No information is given as to source or exact nature of dyes used.)

NaOH (0.1%), Na_2CO_3 (1%), and $NaHCO_3$ (10%) can be successfully applied also after the Löffler stain. Na_2CO_3 (0.5%) and NaOH (0.05%) are also satisfactory after the violet. For best results by method 1 with bovine and human strains use 1 to 5% NaOH, and 3 to 10% NaOH, respectively. For method 2 use saturated $NaHCO_3$ for both bovine and human strains, and 5% Na_2CO_3 for bovine, and 10% for human.

Further studies are being carried on for a possible determination between the various stains.

CLEVELAND, R. and WOLFE, J. M. A differential stain for the anterior lobe of the hypophysis. Anat. Rec., **51**, 409–413. 1932.

Anterior lobes of the hypophysis of the domestic sow, the dog, albino rat, and the rabbit in various physiologic conditions stained by the following method demonstrate four types of cells with greater accuracy and clearness than other stains tried.

Fix in Regaud's solution for 5 days, changing every 24 hours. Transfer to 3% potassium dichromate for 8 days, changing it every 48 hours; place in running water for 24 hours; and dehydrate very slowly. Clear in cedar oil, xylol and embed in 60° paraffin.

Stain 2 to 3 thick sections in Ehrlich's hematoxylin for 3 minutes, rinse in distilled water. Color blue in lithium carbonate and transfer to a 5% solution of dichromate for 3 days, change daily and keep in the dark. Rinse in distilled water and stain for 20 to 30 seconds in 5% erythrosin, pass thru 2 changes of distilled water. Stain in a 2% solution of orange G in 1% solution phosphomolybdic acid for 2 to 3 minutes. Rinse, immerse in 1% anilin blue for 30 to 60 seconds. Rinse, pass thru 95% alcohol, absolute, xylols, and mount. (Only Grübler's stains were used in this procedure.)

Results: Red blood cells bright yellow; connective tissue and blood vessels blue; nucleoli yellow or red. Various glandular cells show various coloration and granules. Fibers or strands of tissue which are found in the posterior lobe stain dark blue, the droplets within the meshes red. The method is reliable and constant.

IMSCHENETZKY, A. Über die Permeabilität der Haut für Trypanblaulösung unter Berücksichtigung der Hautimmunitätstheorie von Besredka. Zts. Exp. Med., **69**, 113–125. 1930

The permeability of the skin to trypan blue was studied and the type of cells involved in the collection of the latter was observed. Trypan blue was chosen as a colloidal stain for percutaneous applications, with the idea that other colloids might prove of therapeutic value.

A 48-hour application of 1% trypan blue, by means of a poultice produces exudative infiltrating inflammation, accompanied by changes of the epithelium and hair follicles in a state of necrosis. These regions, with damaged epidermis and changes in the walls of the hair follicles serve as ports of entry into the lymph system of the corium.

The histiocytes absorb the stain and it accumulates in the cytoplasm as granules. Granules are observed also in certain polymorphonuclear leucocytes, particularly under the epidermis, and occasionally near the hair. The stain is carried to the lymph nodes and there it is deposited in reticulo-stromal cells and in the histiocytes. External application without a poultice stains the upper portions of the horny layer, while the corium remains unstained.

KIRKMAN, I. J. Neutral red as a counterstain for Pal-Weigert sections. Anat. Rec., **51**, 323–326. 1932.

Clear preparations of brain stem sections may be obtained with neutral red as counterstain in which nuclei stand out distinctly in their relation to fiber tracts. The method is based on Ludford's neutral red, modified for the Pal-Weigert procedure.

Counterstain Pal-Weigert's sections in neutral red, (neutral red, Coleman & Bell, 1 g.; distilled water 500 cc.; 1% acetic acid 2 cc.), for 10 to 20 minutes or longer. Rinse in distilled water, differentiate in 95% or absolute alcohol, dehydrate, clear and mount. A longer period of overstaining permits of almost complete differentiation of the background, leaving the glial cells faintly pink, whereas the nerve cells take up the stain brilliantly. Nucleoli, nuclear membranes, and Nissl granules stand out clearly against a pale pink cytoplasm.

The slight acidity of the solution seems to enhance the staining qualities of the dye, helps to overcome the inimical action of the bichromate fixation, and leaves the blueness of the fibers unaltered. The solution was also tested as a straight Nissl stain on Bouin and formalin fixed material and was found to give as clear and distinct a Nissl picture as a section of the material stained by the Huber toluidine-blue method.

LAROCHELLE, F. D. Modification of use of Wright's stain. J. Lab. and Clin. Med., **27**, 818–819. 1932.

Having taken due precautions about the quality and cleanliness of glass, the quality of stains, adjustment of light and filters for the microscope, etc., the H-ion concentration of the water used in diluting Wright's stains is the main requirement for a good blood picture. Fill a gallon bottle with tap water and prepare with it a usual smear stained by Wright's method to determine the reaction of the water. The water used by Dr. LaRochelle is slightly acid and requires 5 to 6 drops of 5% NaOH per gallon; a few slides are stained to find out how many more drops are necessary for best results. In some localities 5 drops may prove too much.

Proceed as follows: Place the air-dried smear over a water glass whose margin must describe an exact horizontal plane if the stain is to flow evenly; float with 30 drops of Wright's stain for 2 or 3 minutes. Dilute with the adjusted water, and after 2 to 3 minutes add 5 or 6 drops of Giemsa's stock solution for 6 to 8 minutes. Giemsa's solution intensifies the basic stain, and this is offset by washing in tap water which is slightly acid. The use of tap water is naturally a great convenience. Dry in air, and examine with liquid petrolatum.

In connection with the last step it should be noticed that the author is located at Springfield, Mass., which is in a region of soft water. In localities where tap water is alkaline (as most common in North America) this technic could not be followed without modification.

PICKARD, R. J., and RICE, C. Method of staining fecal protozoa. J. Lab. and Clin. Med., **27**, 493–494. 1932.

The method here offered is to be used instead of the fresh or iodine fecal preparations. Cysts of *Entamoeba histolytica* (*E. Hartmanni*) are easily mistaken for yeast cells by the older methods.

Fix in freshly made alcoholic Bouin's solution (30 cc. of 80% alcohol saturated with picric acid, 12 cc. of 40% formalin, and 3 cc. of glacial acetic acid), for 1 hour. Harden 5 minutes in 95% alcohol, transfer to 75% alcohol for 5 minutes or until convenient. Wash in 2 changes of water and mordant in 3% iron alum for 15 minutes at 32°C. for the rapid technic, or 12 hours for the longer procedure at room temperature. Wash in distilled water and stain in 1% hematoxylin for 15 minutes at 32°C. for the rapid technic, or 12 hours at room temperature for the other. Wash in water, differentiate in the iron alum, at 32°C. or at room temperature, according to whether the fast or slow method is used. Wash in water, then in 95% alcohol. Clear and examine in creosote oil under oil immersion. For permanent preparations differentiate more carefully; if necessary, wash, dehydrate, clear and mount in balsam as usual.

RETTIE, T. A new method of applying Nile blue as a fat stain. J. Path. and Bact., **34**, 595–596. 1931.

The method is supposed to excel some others because of the fact that all the fat is stained pink so that even small granules are seen unobscured by the blue.

Prepare "Nile pink" as follows: Dissolve 3 g. of Nile blue A in 500 cc. of water, add 3 cc. of H_2SO_4 (concentration not mentioned) and 100 cc. of xylol. Heat the mixture under continuous shaking. The Nile pink dissolves in the xylol, recover by distilling off the xylol. (No information is given as to source or dye-content of the Nile blue A.) Prepare stock solution of Nile pink by dissolving it to saturation in alcohol. This solution will not stain. Mix 2 cc. of the above with 1 cc. of 20% H_2SO_4, 7 cc. of 5% dextrin and 0.2 cc. of 0.5% Nile blue. This solution will contain 0.015% Nile pink in 0.01% Nile blue, 20% alcohol and 2% H_2SO_4. Do not prepare large quantities of solution as there is a slow continuous deposition of the stain. Stain frozen sections ½ to 1 hour, wash in water and mount in glycerol or farrant solution; the fat will show up a bright pink against a gray-blue background.

To demonstrate very fine granules, use the stain without the addition of the Nile blue. To demonstrate fat in fresh or fixed thin layer tissues such as the diaphragm or the omentum, prepare the following solution: 1 cc. of the stock solution, 2 cc. of 20% H_2SO_4, diluted with water to 40 cc. Float the tissue in this solution. The fat alone will be stained pink.

SALAZAR, A. L. and ESTRADA, A. Sur une réaction colloïdale nouvelle de l'hémoglobine. Compt. Rend. Soc. Biol., **109**, 488–490. 1932.

Erythrocytes do not react with ferric tannate if films are properly smeared on clean slides. Blood films smeared on dirty slides show black or grayish corpuscles crowded in dirty, irregular areas. When smears are made on very clean slides with scratches on them, only those erythrocytes react which are in contact with the scratch. Impurities, irrespective of their chemical composition, exhibit the same influence.

The mechanical injury of contact apparently results in a shock which alters the physical or chemical equilibrium of the living erythrocyte. It cannot be questioned but that the reaction is really due to hemoglobin for if the latter is extracted the color fails to appear under otherwise similar conditions.

When erythrocytes are in contact with polymorphonuclear neutrophils they show complete or partial reaction. On the other hand, there are slides where erythrocytes in contact with neutrophils, mononuclear leucocytes and lymphocytes fail to react. This seems to contradict the former impression that the nature of the impurities is of no consequence. The fact that living bodies, such as the neutrophils, etc., produce a lesser shock than mechanical particles is hardly sufficient explanation. It seems rather that the specific action of the polymorphonuclear cells is due to their weak agglutinizing power. The agglutinization produces the shock, and thus renders the reaction possible. The latter takes place when the static condition of the blood is altered in the smear.

STONE, L. S. Selective staining of the neural crest and its preservation for microscopic study. Anat. Rec., **51**, 267–273. 1932.

The mesectoderm and mesentoderm of the neural crest may be differentiated with Nile blue sulfate as a vital stain, the mesectoderm being stained, while the mesentoderm remains unaffected. The staining is based on Vogt's agar method.

Prepare agar sheets by pouring 1 to 2% agar-agar on a glycerined surface. Strip the material off (after setting) and transfer to clean paper for 1 to 2 days, or until dry. Place in 1% Nile blue sulfate for a week (source of dye not mentioned). Rinse the strips in distilled water and deposit on glass surface covered with a film of beeswax. Press flat with finger so they will stick together. Before applying the agar to the embryo, soak it in distilled or tap water for 2 to 24 hours.

Place the strips of agar on the crest of the neural fold in the cranial region of embryos of *Amblystoma*, *Pleurodeles*, or *Rana* in ½ to ¾ normal Ringer's solution for 45 to 60 minutes. A method for the preserving of the stain and yolk-laden cells has been devised.

Fix in Zenker's acetic fluid for 2 hours, and wash under tap for 1 hour. Transfer to 1% phosphomolybdic acid for 1 hour and pass thru 50, 70, 80, 95 and 100% alcohols, each with 0.1% phosphomolybdic acid, at 30 minute intervals. Place in a mixture of 100% alcohol with 0.1% phosphomolybdic acid, and cedar oil. Leave in cedar oil over night. Pass thru three 20-minute paraffin baths and embed. The blue stain remains unaltered, because of the acid. Sections may be freed of paraffin and mounted directly, or counterstained. In the latter case, the alcohols must contain the same percentage of phosphomolybdic acid as those used before embedding.

STRAUSS, A. Über die Bleichung des Melanins. Zts. Wis. Mikr., **49**, 123–125. 1932.

Artificial melanin was shown to dissolve under the action of potassium hydroxide and hydrogen peroxide, or hydrogen peroxide with manganese dioxide. The colorless fluid obtained contains no substances characteristic of the formation of melanin, and is therefore not suitable for chemical studies of the latter. The method proved, however, quite desirable for bleaching histologic preparations.

Paraffin sections of strongly pigmented melano-sarcommetasteses served for material. Experiments at various pH-values proved that stronger alkalinity hastens the bleaching, while the concentration of H_2O_2 is of lesser importance. Thus bleaching is achieved with 30% H_2O_2 with 0.7% KOH within 45 minutes, but in 2 days without the KOH; with 10% H_2O_2 plus 0.7% KOH within 45 minutes, and without the KOH in 2½ days. Manganese dioxide slightly hastens the action when added to a neutral medium, and has almost no effect with an alkaline. Lipofuchsin is also bleached, but hematogenic pigments remain unaltered.

For sections, use a 3% solution of H_2O_2 with 0.5 to 0.7% KOH. If staining is to follow, add approximately 1% Na_2HPO_4 instead of the KOH. Neutralize with 1% acetic acid and rinse in water before proceeding further.

TARKHAN, A. A. The effect of fixatives and other reagents on cell size and tissue bulk. J. Royal Micro. Soc., **51**, 387–399. 1931.

Some of the conclusions obtained from an extensive study of the most important changes produced by fixatives and other reagents as observed from animal tissue are given here. The conclusions are drawn from volumetric study, graphic registration, and histologic examination.

Formol 5 to 10% over periods of 24 to 48 hours does not swell tissues. Its application for longer periods should be avoided because of the toughening effect. Mercuric chloride and picric acid (in saturated aqueous solutions) are powerful shrinking agents; as is absolute alcohol. Strong solutions of chromic acid (1%) and potassium bichromate (4%) are similar in effect. Acetic and trichloracetic acids swell the cells and collagen fibers if used alone. Tissues should be transferred directly to 96% alcohol from these acids instead of water to prevent further swelling.

The capacity for shrinkage of tissues appears to be limited. Thus tissues treated with fixatives that produce but little shrinkage are all the more liable to shrinkage after dehydration, and vice versa. Clearing is productive of a slow and progressive shrinkage, and this has little relation to the fixative used. Xylol and toluol shrink tissues more than benzol, while cedar oil produces the least shrinkage of the four. Paraffin embedding should be cut down to the shortest time possible. Paraffin causes progressive shrinkage, and this is directly proportionate to the temperature.

TSCHETSCHUYEVA, T. Über die Speicherung von Trypanblau in Ganglien verschiedener Gebiete des Nervensystems. Zts. Exp. Med., **69**, 208–219. 1930.

Intravenous and subcutaneous injections of trypan blue solutions in rabbits proved that all the ganglia of various parts of the nervous system accumulate the above mentioned stain. Most intense coloration is attained by the ganglionic cells of the sympathetic ganglia, intermediate by the cerebral, and weakest by the spinal.

Trypan blue is always first taken up by mesodermal cells, later by satellites and ganglionic cells, (that is, when more dye is added). This power of accumulation of the stain depends also upon the stage of a particular cell—the color is most intense in those showing regressive changes.

WETZEL, A. Paraffineinbettung über Terpineol oder Methylbenzoat unter Vermeidung von absolutem Alkohol und Benzol (Xylol, Chloroform) für schwerschneidbare Objekte. Zts. Wis. Mikr., **48**, 360–363. 1931.

The method recommended has been worked out at the Zoölogical Institute at Leipzig and offers special advantages when chitin, yolk, or horny substances come in question.

Methylbenzoate, or preferably terpineol, is used between 96% alcohol and paraffin and absolute alcohol is dispensed with. Prepare the terpineol by dissolving 50 g. in 10 g. of paraffin of 56 to 58° melting point at 45° C.

Transfer tissue from 96% alcohol to mixtures of terpineol or methylbenzoate with 96% alcohol as follows: 1:1 for one hour, or until material sinks; 2:2, and 3:1 for an hour each. Then pass thru three changes of terpineol (or methylbenzoate) alone, or with some $Ca\,Cl_2$ or CaC_2 to remove traces of water or alcohol. (All the liquids except the last can be used over.) High containers are of advantage because the objects sink to the bottom, while the alcohol, if present, may be pipetted off the surface.

Transfer thru three one-hour changes of paraffin and terpineol. Use high containers and suspend the material in the upper portion of the glass, where purer paraffin is insured because of its lower specific gravity. Embed in 52° to 56° pure paraffin.

Methylbenzoate must be colorless, should evaporate slowly and leave no residue. Terpineol should be anhydrous.

BOOK REVIEWS

CHAMBERLAIN, C. J. Methods in Plant Histology. Fifth revised edition. xiv + 416 pp. Univ. of Chicago Press. 1932. $3.25.

This very useful book has been enlarged and very generally revised. The extent to which it has been enlarged is indicated by the fact that the fourth edition consisted of only 343 pages. The additional material has been introduced at various places thruout the book, increasing the length of nearly every chapter. Especially to be noted are the following additions: five pages in the two chapters on staining; six pages in the chapter of the paraffin method; increasing the chapter on photomicrography from eight to 32 pages; two new chapters, Paleobotanical Microtechnic, and Illustrations for Publication. The additions and corrections made in the chapters on staining methods and on the paraffin procedure are especially useful. The writer plainly prefers paraffin to celloidin as an embedding medium, and has made only minor changes in the short chapter describing the celloidin technic. One change of considerable importance is the lengthening of the index, which was a weak part of earlier editions, from three pages to eight.

GAGE, S. H. The Microscope. Ultra-violet (15th) Edition. viii + 190 pp. Comstock Publ. Co., Ithaca, N. Y. 1932.

A new edition of this well-known work will be welcomed by all microscopists. This book has long been regarded as authoritative; with this last revision it is brought up to date and becomes more valuable than ever. As claimed in the preface:

"For this edition the entire work has been reset; out-of-date illustrations replaced, the matter in general revised, and in many places rearranged, and brought up to date. The greatest change in this revision has been the addition of a wholly new chapter (VI) on the Ultra-Violet Microscope and its use. Furthermore, especial emphasis has been put upon what may be properly called 'the physical analysis of structure' by means of the dark-field, the ultra-violet and the polarizing microscope, and the microspectroscope."

While not discouraging the use of stains, the writer is so strongly convinced that other methods of microscopic study can be used to great advantage that he lays much more stress upon the latter than upon the much better known staining procedures. As staining is only one of the ways by which knowledge of structure can be gained, the author contends that all other means available should also be utilized.

GRADWOHL, R. B. H. Laboratory Technic. 462 pp. Gradwohl School of Lab. Technic. St. Louis, Mo., 1932.

A handbook for the laboratory technician, giving methods, formulae, tables, etc., for use in the following lines: clinical pathology, parasitology, hematology, blood chemistry, urine chemistry, toxicology, bacteriology, pathological mycology, serology, basal metabolism and photomicrography. The final section of the book consists of about 35 pages of photomicrographs illustrating the various diagnoses and other points brought out in the text.

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